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Stereochemical assignment of varicosenone, a merosteroid flexible side chain, from the Indonesian sea slug *Phyllidia varicosa* using NMR and DFT-based NMR calculations

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ABSTRACT

A merosteroid with a flexible side chain named varicosenone (**1a**) was isolated from the sea slug *Phyllidia varicosa* collected from Banten, Jakarta, and South Sulawesi, Indonesia. The structure of **1a** was elucidated using 1D and 2D nuclear magnetic resonance (NMR), mass spectrometry (MS) as well as density functional theory (DFT)-based NMR calculations. The relative configuration for the rigid portion (cyclic portion) was evidenced by nuclear Overhauser effect spectroscopy (NOESY) correlation, while the two-chiral centers on the flexible side chain were assessed by statistical comparison (including mean and max absolute, RMS error, and DP4 score) of experimental ^{13}C chemical shifts with the results of the Boltzmann-weighted ^{13}C chemical shifts calculated for each of the four potential stereoisomers. The result suggested preference for two of the four possible stereoisomers (18*R**, 21*S**) and (18*S**, 21*S**). A similar analysis, accounting for the full set of ^{13}C chemical shifts for the system, and only ^{1}H shifts for the flexible side chain of DP4 for ^{13}C and ^{1}H chemical shifts of the portion favor (18*R**, 21*S**) stereoisomer. Therefore, **1a** may have a relative configuration as 8*S**, 9*S**, 10*R**, 13*R**, 14*S**, 17*R**, 18*R**, 21*S**.

INTRODUCTION

Phyllidia varicosa is a species of sea slug or nudibranch in the family Phyllidiidae that often contains defensive allomones. The animal is characterized by yellow and blue–grey color as tuberculate notal ridge and black longitudinal color as foot stripe. It is interesting to note that the color and shape of *P. varicosa* are adopted by the juvenile of sea cucumber *Pearsonothuria graeffei* as Batesian mimicry to survive predators. The nudibranch *P. varicosa* is ecologically known to biosynthesize a toxic compound by warning coloration or mucus secretion. The toxic compound is a result of sequestering specific sponge metabolites either as intact chemical structures or with transformations and then used as a chemical defense by the nudibranch.

A representative group of toxic compounds from *P. varicosa* was reported as nitrogenous sesquiterpenes [1] which can be obtained from different collection sites including Hawaii, Sri Lanka, the Philippines, Japan, and Indonesia. For example, Hawaiian specimens of *P. varicosa* consisted of 9-isocyanopupukeanane [2] and 2-isocyanopupukeanane [3], while Sri Lanka specimens contained 3-isocyanotheonellin [4]. Two molecules were isolated from Philippines specimens of *P. varicosa as* 4α-isocyanogorgon-11-ene and 4α-formamidogorgon-11-en [5]. Japanese and Indonesian specimens contained 10-isocyano-4-cadinene [6] and

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9-thiocyanatopupukeanane [7], respectively. In contrast, only a few steroid compounds are distributed into six species of nudibranch including *Dendrodoris fumata* [8], *Doriprismatica atromarginata* [9], *Phyllidiella pustolosa* [10], *Aldisa smaragdina* [11], *Diaulula sandiegensis* [12], and *Aldisa sanguinea* cooperi [13] have been reported. The presence of steroids in the nudibranch may have a certain role in their metabolisms which are difficult to identify as special chemicals of ecological significance since they are common secondary metabolites in all types of living organisms [14]. However, dendrodoristerol isolated from the Vietnamese nudibranch *D. fumata* showed therapeutic potential against six cancer cell lines with IC_{50} 21.63, 22.22, 24.53, 41.19, 25.34, and 21.59 µM, respectively [8].

In our quest for bioactive compounds from Indonesian marine organisms [15−19], we encountered an active hexane layer of *P. varicosa* with LC_{50} 4.67 ± 0.91 µg/mL against brine shrimp lethality assay and purified the layer to give a merosteroid with a flexible side chain that we named varicosenone (**1a**). The structure includes stereochemical assignment which is the subject of this article.

MATERIAL AND METHODS

General

The ^{1}H and ^{13}C-NMR including correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded on a Bruker Avance III-500 spectrometer. The ^{13}C NMR chemical shifts were deduced by analyzing HSQC and HMBC spectra. The high-resolution electrospray ionization mass spectrometry (HRESIMS) data were recorded on a JEOL JMS T1000GCV spectrometer with 2000 V as the default ionization voltage. The Shimadzu HPLC was used with Prominence LC-20AD, DGU-20A5, SPD-20A with UV detector λ 254 nm using HPLC column Cosmosil 5SL-II (normal silica, 20 mm I.D. × 250 mm). The column chromatography used a Merck silica gel 60 (0.063–0.20 mm), while the analytical thin layer chromatography (TLC) was performed on a Merck silica gel 60 F_{254} visualized with $CeSO_4$. All chemical used were reagent grade as received.

Animal Material

Three specimens of *P. varicosa* (30 g) were collected from Jakarta, Banten, and South Sulawesi, Indonesia, while scuba diving (a depth of 10–15 m). It was then stored in EtOH. The sea slugs were recognized as *P. varicosa* sp. by one of us (Prof. Junichi Tanaka). The identification was also made based on the comparison of specimens with the characteristics of *P. varicosa* reported in Indo-Pacific nudibranchs book [20].

Extraction and Isolation

The newly collected sea slug specimens (wet weight, 30 g) stored in EtOH were extracted three times using MeOH (3 × 50 mL). The four solutions were pooled and concentrated under vacuum, and the resulting residue was partitioned between EtOAc and H_2O. The EtOAc layer was then partitioned between hexane and 90% MeOHaq to give a stronger cytotoxic hexane layer. Purification of hexane layer (111 mg) by column chromatography [normal silica, *n*-Hexane:EtOAc (7:3)] followed by normal silica HPLC [*n*-Hexane:EtOAc (9:1)] gave varicosenone (**1a**) (0.96 mg) as a minor constituent, colorless oil; R_f: 0.71 *n*-hexane:EtOAc (7:3).

Cytotoxicity Assay

The cytotoxicity testing was conducted as previously reported [21]. Eggs of brine shrimp (*Artemia salina*) obtained from the commercial product were incubated in artificial seawater at 25 °C for 24 hours. A total of 10 hatched larvae were placed in a well of 24-well plate with brine (1 mL). The test sample was prepared with a series concentration of 2.5; 5; 25; 100 µg/mL and the plates were kept at 25 °C. DMSO was used as solvent to dissolve the material. The mobility of the larvae was observed for 24 and 48 hours to count number of live larvae. An individual without any motion during the observation was considered as dead. Samples were measured in triplicate. DMSO was used as control negative, while swinholide A, latrunculin A, laulimalide and paclitaxel were used as control positive. The LC_{50} was calculated using IBM SPSS 22 software and expressed in µg/mL together with its standard deviation (SD).

Calculation Study

The computational component was conducted with Spartan'20 software [22] utilizing the default NMR Spectrum protocol, which has shown success in aiding in structural assignment over a broad range of natural products [23]. A

Table 1. Cytotoxic activity against brine shrimp *Artemia salina*.

Component	LC_{50} (µg/mL ± SD)
n-Hexane layer	4.67 ± 0.91
90% MeOHaq layer	30.60 ± 3.95
Swinholide A	0.10 ± 0.04
Latrunculin A	0.17 ± 0.06
Laulimalide	1.79 ± 0.30
Paclitaxel	0.09 ± 0.02

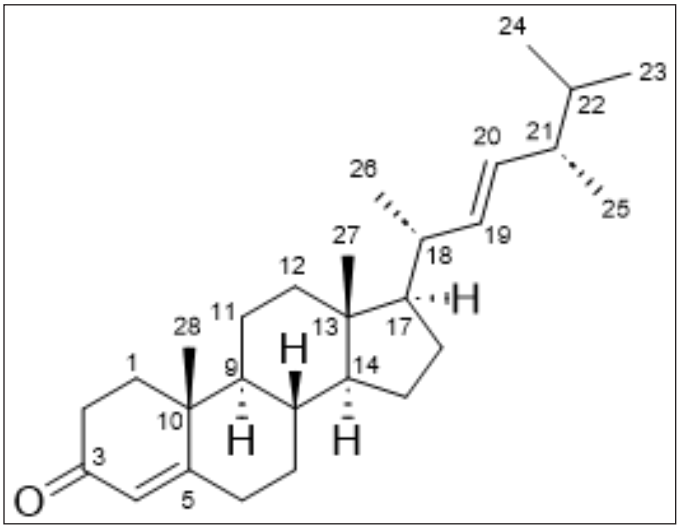


Figure 1. Chemical structure of varicosenone (**1a**)

potential candidate stereoisomer was sketched in 2D, in this case, it was the structure **1a** with C18 and C21 designated as *R* and *S*, respectively. Experimental ^{13}C shifts were assigned based on NMR results. The remaining 3 possible stereo isomers (**1b**, **1c**, and **1d**) were generated, and the collection was submitted to the following computational NMR protocol, generating, refining, and establishing Boltzmann weighted NMR shifts for each candidate: (1) A conformational distribution was performed utilizing the MMFF molecular mechanics model and an initial conformer distribution was established, (2) Further equilibrium geometry calculations were performed using the HF/3-21G model, (3) and (4) energy, and equilibrium geometry calculations were made with the DFT model ωB97X-D/6-31G*, increasing the accuracy of the conformer distribution (and reducing the energy window of the distribution) with each step, culminating in a Boltzmann distribution with energies from (5) ωB97X-V/6-311+G(2df,2p)[6-311G*], and (6) NMR shifts determined from the same ωB97X-D/6-31G* model used in steps 3 and 4. Weighted ^{13}C shifts for each of the 4 stereoisomers (**1a**, **1b**, **1c**, and **1d**), were statistically compared to the experiment. Statistical data including mean and max absolute error, RMS error, and DP4 (%), indicated a preference for two of the four (**1a** and **1b**). Experimental ^{1}H shifts were added to the proton on C17 and the flexible side chain (C18 through C26), and the revised statistical comparison (against experimental shifts) reinforced the preference for **1a** and **1b**. DP4 results (Table 3) suggest **1a** as the likely configuration.

RESULTS AND DISCUSSION

Three specimens of *P. varicosa* were collected at Banten, Jakarta, and South Sulawesi and were exhaustively extracted with EtOH and MeOH. After concentration, the residue was partitioned between EtOAc and H_2O. The EtOAc extract was then partitioned between hexane and 90% MeOH aqueous. The hexane layer showed significant toxicity against brine shrimp lethality assay with LC_{50} 4.67±0.91 µg/mL, while the 90% MeOH aqueous showed LC_{50} > 10 µg/mL (Table 1). Thus, the hexane layer was purified on normal silica gel followed by HPLC using normal silica as a stationary phase to afford varicosenone, **1a** (Fig. 1).

Varicosenone (**1a**) isolated as a minor constituent of *P. varicosa* had a molecular formula $C_{28}H_{44}O$ by HRESIMS indicating seven degrees of unsaturation, *m/z* 397.3442 $[M + H]^+$ (calcd. for $C_{28}H_{45}O^+$ 397.3465). The planar structure of **1a** was determined on the basis of spectral evidence including ^{1}H, ^{13}C, COSY, HSQC, and HMBC as well as by comparing with the synthetic molecule 24-methylcholesta-4,22-dien-3-one as reported by Wright *et al.* [24]. The NMR chemical shift assignment of varicosenone (**1a**) can be seen in Table 2. To confirm the relative configuration of **1a,** especially in the rigid part, the NOESY spectrum of **1a** was measured. The elucidation relative configuration of **1a** especially for cyclic rings was revealed. The presence of two angular methyl groups in the steroid moiety was the same face and adopted *trans* ring junctions as in Figure 2.

The remaining task was to determine the relative configuration of **1a** in the flexible side chain. Of the four

Table 2. NMR Data for varicosenone (**1a**).

# C	δ_C (ppm)[a]	Mult.[b]	δ_H (ppm) (*J* in Hz)[c]
1	35.78	CH_2	1.70, 2.02 m
2	33.84	CH_2	2.36, 2.42 m
3	200.05	C	-
4	123.92	CH	5.72 s
5	171.71	C	-
6	32.88	CH_2	2.26, 2.35 m
7	32.24	CH_2	1.44, 1.82 m
8	35.79	CH	1.52 m
9	53.87	CH	0.91 m
10	38.33	C	-
11	20.94	CH_2	1.42, 1.51 m
12	39.66	CH_2	1.16, 2.00 m
13	42.78	C	-
14	56.13	CH	1.01 m
15	24.15	CH_2	1.06, 1.57 m
16	28.67	CH_2	1.25, 1.67 m
17	51.55	CH	1.52 m
18	39.98	CH	2.00 m
19	135.87	CH	5.15, dd (*J* = 15.7; 7.2)
20	132.64	CH	5.18, dd (*J* = 15.7; 7.2)
21	42.77	CH	1.86 m
22	33.20	CH	1.46 m
23	19.43	CH_3	0.81, d (*J* = 6.9)
24	19.97	CH_3	0.84, d (*J* = 6.9)
25	17.71	CH_3	0.91, d (*J* = 6.9)
26	20.93	CH_3	1.00, d (*J* = 6.5)
27	12.21	CH_3	0.72 s
28	17.37	CH_3	1.18 s

[a]^{1}H NMR ($CDCl_3$, 500 MHz), [b]multiplicity was determined using HSQC experiment, [c] ^{13}C NMR ($CDCl_3$, 125 MHz)

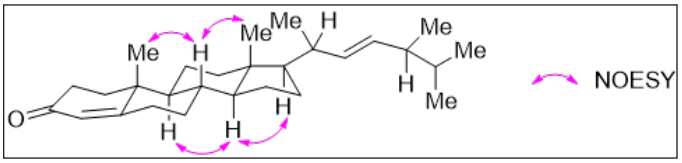
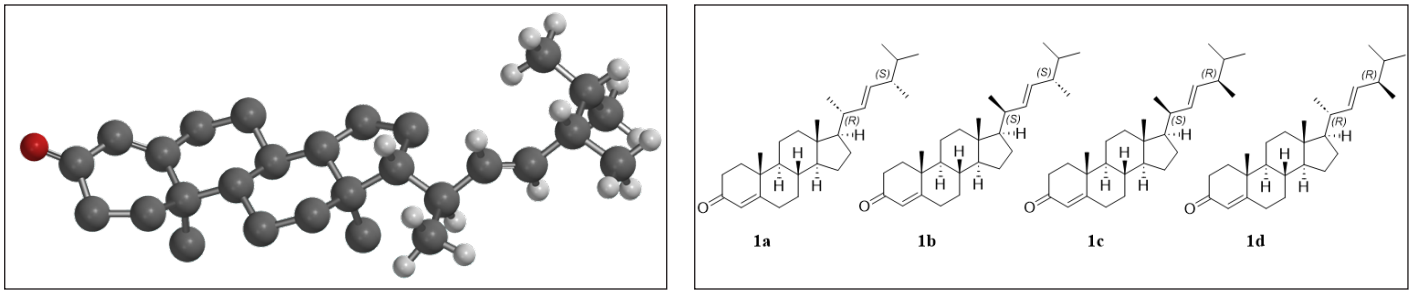


Figure 2. Relative configuration of **1a** especially in cyclic rings revealed by elucidation of NOESY spectrum.

1a–**1d** possibilities (Fig. 3B), we were able to narrow into two possibilities of stereoisomers by comparing experimental results with calculated results using DFT-based NMR calculations and the NMR Spectrum protocol [23] in Spartan'20 software. The DP4 C (%) was 35.6 for **1a** and 29.9 for **1b**, respectively, while **1c** and **1d** gave DP4 C (%) 18.2 and 16.3, respectively. While not conclusive, there did appear a preference toward either **1a** or **1b**. Experimental ^{1}H shifts were added for the proton on C17 and flexible portion from C18 to C26 which showed



A B

Figure 3. 3D image of the system (hydrogens visible where experimental proton shifts were included (A). The four possibilities of stereoisomers **1a−d** in the flexible side chain (B)

Table 3. DP4 summary for C (%), H (%), H+C (%) for **1a**, **1b**, **1c**, and **1d.**

Compound	DP4 C (%)*	DP4 H (%)**	DP4 (H+C) (%)**
1a (18*R**, 21*S**)	35.6	64	68
1b (18*S**, 21*S**)	29.9	36	32
1c (18*S**, 21*R**)	18.2	0.14	0.076
1d (18*R**, 21*R**)	16.3	0.19	0.094

*The DP4 C (%) includes analysis of all carbon centers.
**The DP4 H (%) and DP4 (H+C) (%) compare the proton data or proton + all carbon data, the proton data considered is only for the flexible portion from H17 on the steroid scaffold and the full flexible side chain from C18 to C26 as shown in Figure 3A.

a high degree of uncertainty in relative configuration on the two chiral carbons (C18 and C21), and statistical analysis with DP4 scores were again performed. Results showed that while both **1a** or **1b** remained viable candidates, the likelihood of **1c** and **1d** was significantly reduced (statistically insignificant). Further, both DP4 H (%) and DP4 (H+C) (%) favored the same stereoisomer, **1a**, by approximately 2 to 1, 36% and 32% for the 18*S**, 21*S** configuration and 64% and 68% for the 18*R**, 21S* stereoisomer. The results of the four stereoisomers can be seen in Table 3. Because of the small amount of material, we were not able to confirm the cytotoxicity for the pure material.

Although compound **1a** has been reported as a commercial compound [24] and isolated from *P. pustulosa* [10]. The relative configuration of **1a** remained to be established, especially in the flexible portion. Previous structure determination including stereochemical elucidation for this type of steroid has been done using chemical correlation of the related compounds [25]. There was no stereochemical determination for **1a**. To the best of our knowledge, the stereochemical determination of the steroid class compound featured with a flexible side chain using DFT-based NMR calculations is a new approach. Biosynthetically, **1a** is newly grouped as a merosteroid or more generally as a meroterpenoid because the compound has a mixed biosynthetic pathway [26]. Extra carbon atoms at C21 arose through the *S*-adenosylmethionine mechanism [27]. The side chain of **1a** containing methyl group at C21 was unique providing significantly improved bioactive properties such as selectivity, solubility, half-life, and binding affinity of small molecule drugs [27]. In addition, the chemical metabolite **1a** was discovered for the first time in the genus of *Phyllidia* mollusk.

CONCLUSION

In conclusion, our effort to isolate a minor constituent of *P. varicosa* gave varicosenone **1a**, a merosteroid in a minute amount. The planar structure was known, while the stereochemical determination was new. The relative configurations of cyclic portion **1a** were determined using the NOESY spectrum, while the flexible portion was elucidated using DFT-based NMR calculations for the possible stereoisomers for the two-chiral centers in the flexible portion as 18*R**, 21S*. Therefore, the relative configuration of **1a** was 8*S**, 9*S**, 10*R**, 13*R**, 14*S**, 17*R**, 18*R**, 21*S**. Varicosenone **(1a)** was discovered for the first time in the genus of *Phyllidia*.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NH and JT. Performed the experiments: NH, BSN, WSO, TAT, HDY, FFD, DTR, AS, AM, and JT. Analyzed the data: NH, BSN, WSO, HDY, FFD, AM, and JT. Wrote the paper: NH, WSO, and JT.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exist.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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